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# Synthesis and Evaluation of Pseudolipids To Characterize Lipase Selectivities



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Esters of the acetonide of glycerol, (2,2-dimethyl-1,3-dioxolan-4-yl)methanol, hydrolyzed under lipase catalysis with rates comparable to, or greater than, the corresponding triglycerides. They appear to be better substrates for evaluating fatty acid selectivity than the methyl esters that have been frequently used. By contrast, triglycerides that have one or two ester groups replaced to form *n*-alkyl ethers are much slower to hydrolyze. Stereoselection in hydrolysis of esters of the acetonide by several lipases was examined, and significant stereoselectivity was consistently observed.

Lipases, triacylglycerol hydrolases, are employed for a wide range of purposes from fat solubilization to cheese flavor development (Posorski, 1984). Currently considerable interest resides in interesterification, a process that potentially converts a surplus natural triglyceride into a material with more desirable physical properties, e.g., palm oil into cocoa butter (Nielsen, 1985). Greater fundamental understanding of these enzymes is of paramount importance for successfully arriving at new processes using agricultural surplus as feed stock. The study of lipases, however, has been made difficult by the nature of the substrates. Triglycerides present the enzyme with more than one reaction site and yield products, diglycerides and subsequently monoglycerides, that are also subject to enzymatic hydrolysis. In addition, the acyl groups of these partially hydrolyzed triglycerides undergo nonenzymatic internal migration that can be competitive with hydrolysis (Buchnea, 1967). Although a number of alternatives have been used to cope with these problems, determination of lipase fatty acid selectivity, triglyceride positional selectivity, and stereoselectivity are still subject to some ambiguity. We undertook the synthesis and evaluation of a selection of monoacid esters in order to uncover compounds that might serve as pseudolipid substrates. In this process we developed a generally useful procedure for the preparation of 2-alkylglycerols and synthesized variously substituted monoester diethers and monoether diesters of glycerol, and we suggest a method for measuring the stereoselectivity of lipases in triglyceride hydrolysis, indicating that lipases may be stereoselective in reactions with their natural substrates.

## EXPERIMENTAL SECTION

$^1H$ and $^{13}C$ NMR spectra were obtained using a JEOL JNM-GX 400 FT NMR spectrometer employing deuteriochloroform as the solvent with tetramethylsilane as the internal standard. Infrared spectra were obtained on a Perkin-Elmer Model 1310 spectrophotometer using 3% solutions in $CCl_4$. Mass spectra were obtained on a Hewlett-Packard HP-5995 GC/MS system employing an OV-1 column (0.25 mm (i.d.) × 30 m) and isobutane and a Finnigan Model 4500 GC/MS system using a DB-1 wide-bore column (0.32 mm (i.d.) × 30 m) and $NH_3$.

Gas–liquid chromatography was performed with a Shimadzu GC-Mini 2 instrument with FID using a nonpolar capillary column (0.25 × 30 m) with a 50:1 split ratio and He carrier gas at temperatures indicated below. High-performance liquid chromatography was accomplished with a Perkin-Elmer Series 2-LC pump using two Supelco LC-18 columns (0.63 cm × 15 cm) in series with solvents and flow rates as indicated below and a Waters RI detector.

Solvents were reagent or HPLC grade. The (2,2-dimethyl-1,3-dioxolan-4-yl)methanol was converted to its esters by standard procedures, as were phenylmethanol, cyclohexylmethanol, cyclohexylethanol, and cyclopentylmethanol. These compounds were distilled and characterized for purity by GLC; spectral data were consistent with assignments. Consistent $^{13}C$ NMR data were accumulated for all compounds described below. Since complete assignments were not made, only shifts for diagnostic downfield (C—O, C=O) signals are given. Thin-layer chromatographic data are collected at the end of this section for convenience. Specific lipases employed and manufacturers are as follows: *Candida rugosa*, Enzyme Development Co., New York, NY; *Aspergillus niger* K., Amano Co., Troy, VA; *Mucor miehei*, Amano-MAP, also Gist-Brocades-S, Charlotte, NC, also NOVO-L, Wilton, CN; *Rhizopus delemar*, Tanabe Co., Marlborough, NJ; porcine pancreatic lipase, Sigma Chemical Co., St. Louis, MO.

## SYNTHESIS

**(2,2-Dimethyl-1,3-dioxolan-4-yl)methyl *n*-Octyl Ether (1a).** Sodium hydride (7.0 g of 50% in oil, 0.16 mol) was freed of oil by washing with hexane under nitrogen. Tetrahydrofuran (THF) that had been dried by distillation from lithium aluminum hydride was injected (100 mL), and the acetonide of glycerol (Jensen and Pitas, 1974) (15.8 mL, 0.12 mol) was added as a solution in THF (50 mL) while the reaction mixture was stirred and cooled to 0–10 °C. The resulting mixture was stirred without cooling for 1 h, and then 1-bromooctane (17.2 mL, 0.10 mol) was injected. The mixture was warmed to 50 °C overnight and then worked up by dilution with water and extraction with ether in the usual manner. The product was distilled to give 17.9 g (73.4%) of **1a**: bp 92–93 °C (0.4 mm); IR 1125 cm% $n^{25}_D$ 1.4314.

**(2,2-Dimethyl-1,3-dioxolan-4-yl)methyl Benzyl Ether (1b).** Compound **1b** was prepared as described for **1a** with benzyl bromide to give 91% yield; bp 91–93 °C (0.1 mm) [lit. bp 95–97 °C (0.2 mm) (Snowden and Fisher, 1941)].

**Glycerol 1-*n*-Octyl Ether (2a).** Compound **1a** was treated with boric acid (1 g/g of ether per 5 mL of $H_2O$) under reflux overnight. The product was extracted into ether and worked up to give **2a** (66%): bp 120–123 °C (0.5 mm); IR 3450, 1125 $cm^{-1}$; $n^{25}_D$ 1.4498; $^{13}C$ NMR 64.30, 70.51, 71.89, 72.51 ppm; CIMS ($C_4H_{10}$) $m/z$ 205 $(M + H)^+$; lit. bp 248 °C, $n^{20}_D$ 1.4351 (Ulbrich et al., 1964).

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**Glycerol 1-Benzyl Ether (2b).** Compound **2b** was similarly prepared: bp 126–130 °C (0.1 mm) [lit. bp 138–139 °C (0.3 mm) (Snowden and Fisher, 1941)]; $n^{25}_D$ 1.5229; $^{13}C$ NMR 63.98, 70.75, 71.68, 73.51 ppm.

**Glycerol 3-Octanoyl 1-*n*-Octyl Ether (3).** Compound **2a** (2.04 g, 10 mmol) was treated with octanoyl chloride (2.6 mL, 15 mmol) and pyridine (1.2 mL, 15 mmol) in $CHCl_3$ (50 mL), cooled in ice, and then allowed to stand at ambient temperature overnight. After the usual workup procedure, the crude product that was primarily a mixture of **3** and bisadduct **4** was purified by column chromatography on silica gel. Compound **4** eluted with 5% ethyl acetate–hexane, and **3** eluted with 10% ethyl acetate. The following spectral data are for **3**: IR 3580, 1735, 1125 $cm^{-1}$; $n^{25}_D$ 1.4419; $^{13}C$ NMR 65.44, 68.88, 71.43, 71.80 ppm.

**Glycerol 2,3-Dioctanoyl 1-*n*-Octyl Ether (4).** Compound **4** was obtained as described above and also directly by using excess acid halide and pyridine. After column chromatography, **4** was obtained in 82% yield: bp 205–215 °C (0.35 mm) bulb to bulb; IR 1740, 1125 $cm^{-1}$; $n^{25}_D$ 1.4440; $^{13}C$ NMR 62.80, 68.96, 70.09, 71.77 ppm; CIMS, $m/z$ 474 ($M + NH_4^+$).

**Glycerol 1-Benzyl 2,3-Di-*n*-octyl Triether (5).** Glycerol 1-benzyl ether (12.0 g, 65.9 mmol) was converted to a disodio salt with NaH (6.7 g, 139 mmol) in THF as described above. Reaction with 1-bromooctane (23.9 mL, 138 mmol) was enhanced by addition of hexamethylphosphoric triamide (HMPT) (20 mL). Hexamethylphosphoric triamide is a cancer suspect agent and an irritant! The product was worked up in the usual manner, and TLC indicated minor amounts of mono- and dialkylated products (53% yield of **5**). Column chromatography provided a pure sample that was eluted with 10% ethyl acetate–hexane (1.2 g from 1.3 g of crude): bp 220 °C (0.5 mm) bulb to bulb; IR 1125 $cm^{-1}$; $n^{25}_D$ 1.4720; $^{13}C$ NMR 70.43, 70.63, 70.83, 71.67, 73.39, 78.00 ppm; CIMS ($C_4H_{10}$), $m/z$ 407 $(M + H)^+$.

**Glycerol 1,2-Di-*n*-octyl Ether (6).** Triether **5** (12.8 g, 31.5 mmol) was treated with sodium (13 g, 0.57 mmol) in 10 mL of absolute ethanol. Ethanol was periodically added until the refluxing mixture no longer contained metallic sodium. Acetic acid (35 mL) was slowly added to the cooled reaction mixture; the mixture was suction filtered and the filtrate was concentrated. The residue was partitioned between $H_2O$ and ether and worked up. Column chromatography using silica gel (50 g) afforded 5.7 g (58%) of **6** eluted with 15–20% ethyl acetate–hexane: bp 162–164 °C (0.5 mm) [lit. bp 150–155 °C (0.2 mm) (Paltauf and Spener, 1968)]; $n^{25}_D$ 1.4456; IR 3550, 1125 $cm^{-1}$; $^{13}C$ NMR 62.69, 70.25, 70.66, 71.63, 78.37 ppm; CIMS ($C_4H_{10}$), $m/z$ 317 $(M + H)^+$.

**Glycerol 3-Octanoyl 1,2-Di-*n*-octyl Ether (7).** Diether **6** (1.60 g, 5.1 mmol) was treated with octanoyl chloride in pyridine in the usual fashion (1 h, 60 °C) and worked up to give, after column chromatography, 2.26 g (ca. 100%): bp 205–215 °C (0.35 mm); IR 1735, 1125 $cm^{-1}$; $n^{25}_D$ 1.4442; $^{13}C$ NMR 63.74, 70.22, 70.54, 71.67, 76.48, 173.62 ppm; CIMS, $m/z$ 460 ($M + NH_4^+$).

**Glycerol 1,3-Di-*n*-octyl Ether (8a).** Sodium hydride (14.4 g of 50% in mineral oil, 0.30 mol) was freed of oil as described above, and THF (100 mL) was added. Then, 1-octanol (47 mL, 0.30 mol) was added dropwise, maintaining the temperature below 25 °C. The resulting mixture was stirred for 1 h without external cooling. Dry NaI (1 g) and epichlorohydrin (7.8 mL, 0.10 mol) were added, and a mild exothermic reaction ensued that was controlled by swirling the reaction mixture in an ice bath. The mixture was then warmed to 50 °C overnight to complete the reaction. The product was obtained by partitioning between $H_2O$ and hexanes and, after the usual processing, was distilled to give 20.5 g [bp 60–70 °C (0.5 mm)] of recovered 1-octanol, 2.1 g of intermediate material, and 9.49 g (30%) of **8a**: bp 157–163 °C (0.5 mm); IR 3550, 1125 $cm^{-1}$; $n^{25}_D$ 1.4455; $^{13}C$ NMR 69.34, 71.57, 71.86 ppm.

**Glycerol 1,3-Dibenzyl Ether (8b).** Sodium benzyl oxide was prepared in benzyl alcohol from NaH in the manner described above for **8a** and allowed to react with epichlorohydrin in the presence of added NaI to give, after workup, **8b** (71.7%): bp 176–190 °C (0.5 mm); IR 3560, 1100 $cm^{-1}$; $n^{26}_D$ 1.5446; $^{13}C$ NMR 69.53, 71.26, 73.40 ppm; EIMS, $m/z$ 272 ($M^+$), 254 ($M^+ - 18$), 181 ($M^+ - CH_2C_6H_5$), 107 ($C_6H_5CHOH^+$), 91 ($C_6H_5CH_2^+$).

**Glycerol 2-Octanoyl 1,3-Di-*n*-octyl Ether (9).** Diether **8a** was esterified as described above to give **9** quantitatively after column chromatography (8% ethyl acetate–hexane): IR 1735, 1125 $cm^{-1}$; $^{13}C$ NMR 69.23, 71.29, 71.57 ppm; CIMS, $m/z$ 460 ($M + NH_4^+$).

**Glycerol 1,3-Dibenzyl 2-*n*-Octyl Triether (10).** Diether **8b** (8.00 g, 29.4 mmol) was added to lithium diisopropylamide that had been prepared from *n*-butyllithium (13.6 mL of 2.6 M in hexane) and diisopropylamine (5.0 mL, 35.3 mmol) in dry THF (40 mL) under nitrogen. To this solution were added 1-bromooctane (6.1 mL, 35.3 mmol) and HMPT (13 mL), and the mixture was stirred at 25 °C for 16 h. This mixture was then diluted with 2 N HCl and extracted with hexane. Column chromatography provided 3.5 g (31%) of **10**, eluting with 5% ethyl acetate–hexane: bp 205–210 °C (0.4 mm); IR 1125 $cm^{-1}$; $n^{26}_D$ 1.5060; $^{13}C$ NMR 70.08, 70.57, 73.30, 77.87 ppm; CIMS ($C_4H_{10}$), $m/z$ 385 $(M + H)^+$.

**Glycerol 2-*n*-Octyl Ether (11).** Triether **10** was treated with sodium/ethanol as described above to give **11** (53%): bp 114–117 °C (0.2 mm); IR 3550, 1125 $cm^{-1}$; $n^{26}_D$ 1.4496; $^{13}C$ NMR 62.17, 70.23, 79.54 ppm.

**Glycerol 1-Octanoyl 2-*n*-Octyl Ether (12) and Glycerol 1,3-Dioctanoyl 2-*n*-Octyl Ether (13).** The mono- and diester derivatives of **11** were obtained by esterification using 1.5 equiv each of octanoyl chloride and pyridine in $CHCl_3$ at 25 °C for 16 h. The crude product was obtained and purified as for **3** and **4** to give samples of **12** and **13**. **12**: bp 185–195 °C (0.4 mm) bulb to bulb; IR 3580, 1735 $cm^{-1}$; $n^{26}_D$ 1.4460; $^{13}C$ NMR 62.05, 62.40, 70.57, 77.70 ppm; CIMS, $m/z$ 348 ($M + NH_4^+$). **13**: bp 220 °C (0.4 mm) bulb to bulb; IR 1735 $cm^{-1}$; $^{13}C$ NMR 63.07, 70.69, 75.20 ppm; CIMS, $m/z$ 474 ($M + NH_4^+$).

**Lipolyses.** *Substrate Reactivity Measurements.* The procedure followed here has precedent in earlier comparisons of selectivity made by Brockerhoff for porcine pancreatic lipase (Brockerhoff, 1968). The candidate ester (0.60 mequiv) was weighed into a vessel to which was added 10 mL of 0.05 N tris(hydroxymethyl)aminomethane (Tris) buffered at pH 7.00 (pH 8.00 for porcine pancreatic lipase) containing 0.01 M $CaCl_2$ and 25 drops of 10% gum arabic solution. The mixture was sonicated for 10 s, and the freshly prepared enzyme solution was added. The concentration of enzyme and volume of added solution were adjusted from initial values of 1 mL of 1 mg/mL so as to provide a measurable velocity using pH-stat conditions with 0.2 N NaOH during a 10-15-min period. Pseudo-first-order rate values were calculated from initial slopes. Emulsions were difficult to reproduce exactly, and compounds evaluated had different abilities to hold the emulsion. Nevertheless, qualitative comparisons could be reliably made.

*Competitive Lipolysis of 7 and 9.* The diether esters **7** and **9** (132 mg each, 0.600 mequiv) were sonicated with

10 mL of 0.05 N Tris buffer at pH 7.00 containing $CaCl_2$ (0.01 M) and 25 drops of 10% gum arabic for 10 s. *C. rugosa* lipase powder was added in an amount that the relative reaction rates calculated indicated would be appropriate (5–20 mg), and the mixture was stirred for 24 h at 30 °C. Free fatty acid titration to pH 9.50 after dilution with 100 mL of ethanol against a suitable control enabled determination of fraction conversion, $C$, for the sample. The samples were then extracted with hexane to recover the organics, and these were then examined at 10% acetone solutions by HPLC. Area measurements were normalized with a suitable control extraction. With $CH_3CN$ at 1.0 mL/min $k$ values were 2.00 and 2.24 for the hydrolysis products **6** and **8a**, respectively ($\alpha$ = 1.12, resolution 0.8). The percent excess (% **6** − % **8a**) is related to the ratio of *residual* esters **7** and **9** by

$$\%\ \text{excess}\ (\mathbf{9}-\mathbf{7}) = \frac{\%\ \text{excess}\ (\mathbf{6}-\mathbf{8a}) \times C}{1-C}$$

An extension of the relationships that were derived for enzymatic resolution of racemates (Martin et al., 1981; Wang et al., 1984) was then employed to obtain a value of rate ratio that offers a means by which to characterize the position selectivity of a given enzyme preparation:

$$R = \frac{\ln (1-C)(1-\text{fraction excess, starting matl})}{\ln (1-C)(1+\text{fraction excess, starting matl})}$$

Specifically, **7** and **9** reacted in the presence of *C. rugosa* lipase to 25.4% conversion ($C$ = 0.254). The ratio of products was 70:30, a 40% excess of the primary alcohol **6**. The excess of **9** over **7**, therefore, was 13%; i.e., the fraction excess was 0.13, and the calculated rate ratio, $R$, was 2.6.

A similar experiment involved esterification of equimolar quantities of **6** and **8a** in hexane to which was added 1 μL of the Tris solution, 20 mg of lipase powder, and 40 mg of Celite to aid in maintaining the dispersion (as water is generated the protein coagulates). The determination of $C$ and ratio of starting materials at that point in the reaction again indicated the faster reaction of the primary alcohol ($R$ = 4.9).

*Stereoselectivity of Lipases in Hydrolysis of Acetonide Esters 14*. Ester **14** (4.00 mmol) was suspended in 10 mL of Tris as described above (pH 7.00 for all fungal lipases and pH 8.00 for porcine pancreatic lipase); an amount of enzyme was added as a powder (20–35 mg); the mixture was briefly sonicated and then allowed to react with pH-stat conditions at ambient temperature to 40–50% conversion. Depending upon the reactivity of the substrate and the amount of enzyme, the time required was 2–4 h. The mixture was immediately diluted with 2 vol of $H_2O$ and extracted thoroughly with ether. The organic phase was washed with water several times to remove the hydrolysis product, the acetonide of glycerol. The ethereal layer was dried and concentrated, and the recovered **14** (no longer racemic) was transesterified (0.5 g of $NaOCH_3$, 10 mL of $CH_3OH$, 1 h, 25 °C). Acetic acid (0.5 mL) was added, and the mixture was stripped to dryness. The residue was triturated with ether to recover that portion of unlipolyzed ester as alcohol. This alcohol was oxidized to the acid with Jones reagent (Fieser and Fieser, 1967) in acetone (ice bath), and the acid was recovered by partitioning the reaction mixture between brine and ether. The ethereal layer was dried and concentrated, and the acid was converted to the acid halide (0.2 mL of $SOCl_2$, 20 μL of DMF, 20 mL of anhydrous ether, 1 h, 25 °C). The mixture was diluted with 20 mL of hexane, passed through $Na_2SO_4$, concentrated, and then added to a solution of 100 μL of (*S*)-α-methylbenzylamine and 300 μL of triethylamine in $CH_2Cl_2$ (10 mL) to form the amide. The organic phase was washed with 2 N HCl to remove excess amine, and the crude diastereomeric amides were recovered and employed directly for analysis by GLC [SPB-1 column, 210 °C], $k$ (configuration): 2.04 (*S,S*), 2.23 (*R,S*), $\alpha$ = 1.094. The elution orders were determined with a sample of the (*S*)-acetonide of glycerol (Fluka, Hauppauge, NY). The values of $C$ and enantiomeric excess of recovered starting material were used to calculate the enantiomeric ratio by the relationship described above.

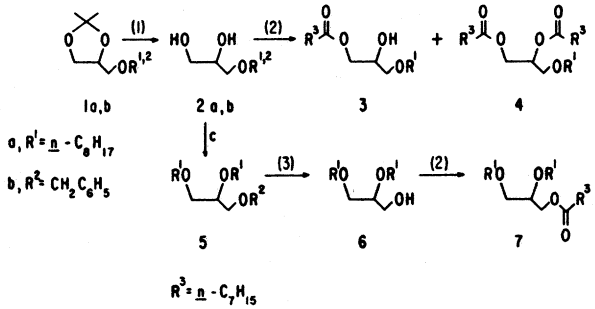
*Thin-Layer Chromatography*. Chromatograms were obtained with use of Analtech silica gel GF plates, 250, developed with solvent indicated, and visualized with iodine (Table I).

**Table I. $R_f$ Values (TLC)**

| compd | substituent[a] 1 | 2 | 3 | TLC solvent A[b] | B[c] | C[d] |
|---|---|---|---|---|---|---|
| **2a** | OR | OH | OH | 0.41 | 0.10 | |
| **11** | OH | OR | OH | 0.41 | 0.10 | |
| **6** | OR | OR | OH | 0.83 | 0.54 | |
| **12** | OH | OR | OX | 0.80 | 0.43 | |
| **3** | OR | OH | OX | 1.0 | 0.62 | |
| **8a** | OR | OH | OR | 1.0 | 0.67 | |
| **5** | OR′ | OR | OR | | | 0.63 |
| **7** | OR | OR | OX | | | 0.71 |
| **9** | OR | OX | OR | | | 0.72 |
| **4** | OR | OX | OX | | | 0.65 |
| **15** | OX | OX | OX | | | 0.68 |

[a] R = *n*-octyl (octyl ether); X = *n*-octanoyl (octanoate ester); R′ = benzyl. [b] A = $CHCl_3$ (72.5), acetone (25), methanol (2.0), acetic acid (0.5). [c] B = $CHCl_3$ (96), acetone (4). [d] C = hexane (85), ethyl acetate (15).

**Scheme I. Synthesis of Glycerol 1-Octyl and 1,2-Dioctyl Derivatives[a]**

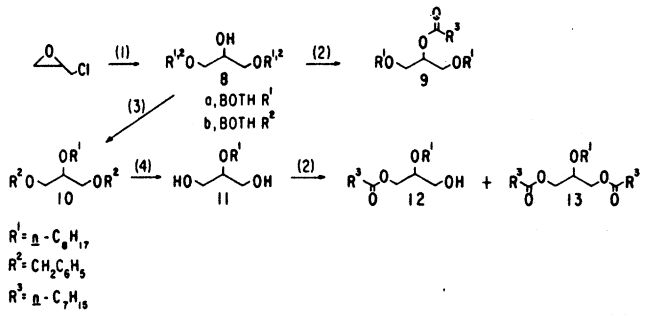



[a] Key: (1) $H_3BO_3$, $H_2O$, Δ; (2) $C_7H_{15}COCl$, py; (3) NaH, $C_8H_{17}Br$; (4) Na, EtOH.

## RESULTS AND DISCUSSION

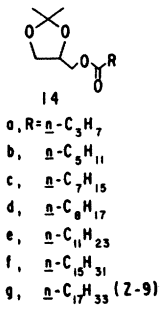
**Synthesis.** The acetonide derivative of glycerol (Scheme I, R = H) was converted to its sodium salt in THF and allowed to react with 1-bromooctane to form the 1-*n*-octyl ether **1a**. The choice of an 8-carbon chain was based on the observation that virtually all lipases examined for fatty acid selectivity show significant reactivity toward the octanoic acid residue. Thus, we could expect reasonable reaction rates for trioctanoin and should seek compounds of chain length that would serve as "pseudotrioctanoins". The acetonide was cleaved with boric acid, producing 1-*O*-octylglycerol (**2a**) that was then treated with 1 or 2 equiv of octanoyl chloride and pyridine, giving either a mixture rich in the 1,3-disubstituted glycerol **3** or the fully substituted glycerol **4**. Both **3** and **4** were obtained pure by column chromatography. Alternatively, the benzyl

**Scheme II. Synthesis of Glycerol 2-Octyl and 1,3-Dioctyl Derivatives**[a]



[a] Key: (1) $R^{1,2}$ = ONa, $R^{1,2}$ = OH; (2) $C_7H_{15}COCl$, py; (3) NaH, $C_8H_{17}Br$; (4) NaH, EtOH.

**Chart I. Esters of Glycerol 1,2-Acetonide**



14

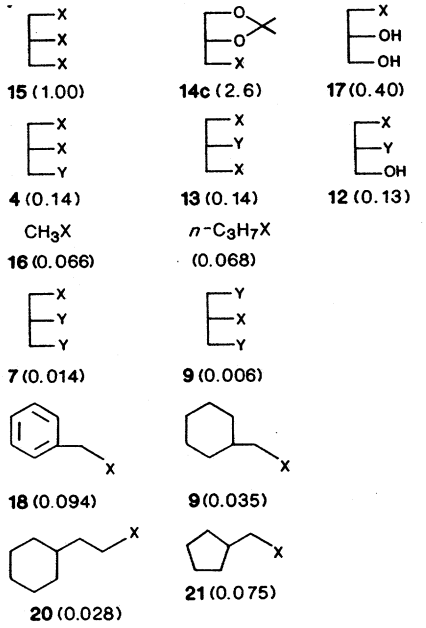
a, R = n-$C_3H_7$
b, n-$C_5H_{11}$
c, n-$C_7H_{15}$
d, n-$C_8H_{17}$
e, n-$C_{11}H_{23}$
f, n-$C_{15}H_{31}$
g, n-$C_{17}H_{33}$ (Z-9)

ether of the acetonide of glycerol was transformed to 1-*O*-benzylglycerol (**2b**), and this was converted to a disodio derivative and dioctylated to give **5**. Treatment with sodium/ethanol produced 1,2-di-*O*-octylglycerol (**6**) that was then esterified to give diether ester **7**.

In order to obtain unique substitution at the 2-position, epichlorohydrin was allowed to react with excess sodium octyl oxide in 1-octanol (Scheme II). Fractional distillation gave the 1,3-dioctyl ether of glycerol (**8a**) that was esterified with octanoyl chloride, producing **9**. Reaction of epichlorohydrin with excess sodium benzyl oxide gave 1,3-di-*O*-benzylglycerol (**8b**). Compound **8b** was converted with NaH and 1-bromooctane to the triether **10**. Reaction of **10** with sodium/ethanol provided 2-*O*-octylglycerol (**11**) that was then esterified under controlled conditions to produce the 1-ester 2-ether **12** and the 1,3-diester 2-ether **13**. Column chromatography was generally required for final purification of these materials. The procedures described should be applicable to a wide range of structural types. Although a number of syntheses of glycerol alkyl ethers have been reported (Paltauf and Spener, 1968), we are not aware of an effort to describe a complete set of ether esters. The preparation of 2-*O*-octylglycerol from epichlorohydrin appears to be superior to a method employing dihydroxyacetone (Barry and Craig 1955). Finally, esters of the acetonide of glycerol, **14a–g** (Chart I), were synthesized in the manner described by Jensen and Pitas (1974) from the alcohol and acid in benzene with catalysis by *p*-toluenesulfonic acid, and other esters that were evaluated either were used as purchased or were synthesized from commercially available alcohols by the usual methods and purified by distillation.

**Evaluation of Compounds.** *O*-Alkylglycerols have been previously employed as "pseudolipids", for example, in studies of intestinal fat absorption (Paltaul, 1969). In this instance the focus was on a suitable alternate substrate with which to characterize lipase activity. The criteria we adopted were that the candidate would be a monoacid ester, thereby avoiding additional reaction centers on the

**Table II. Relative Reactivity of Octanoate Esters in the Presence of *C. rugosa* Lipase**[a]




[a] X = *n*-octanoyloxy (octanoate ester); Y = *n*-octyloxy (*n*-octyl ether).

substrate and producing a product that would not itself react. In addition, the substrate should be liquid at, or near, room temperature to avoid the further complication of a solid–liquid interface during lipolysis. Lipolyses are two-phase reactions that reportedly derive special benefit from the oil–water interface (Entressangles and Desnuelle, 1968). Emulsion reproducibility is a concern for those who study lipase-catalyzed hydrolyses. If the substrates are liquified and the same procedure is employed in a specific study, the results are reproducible. The most generally accepted criterion for substrate specificity is $k_{cat}/k_M$. The $k_M$ value, however, has been shown to vary with emulsion surface area (Benzonana and Desnuelle, 1965) and for this reason we felt that a more rapid screening of compounds could be effected by comparing initial reaction velocities by a selected protocol (see the Experimental Section). Arbitrarily we decided that an ester was a reasonable substrate if its apparent reaction rate was at least 5–10% of the corresponding triglyceride. Also, we wanted to examine a selection of commercially available lipases. None of these is available as a pure homogeneous protein, and a more comprehensive kinetic study would have been of limited value without that purity.

The reaction velocities relative to trioctanoin (**15**) and normalized for a single acid residue in reactions involving the lipase preparation from the fungus *C. rugosa* (formerly *Candida cylindracea*) are given in Table II. It was noteworthy that methyl octanoate (**16**) was much less reactive than trioctanoin; the reactions of methyl esters frequently serve as the basis for estimating fatty acid selectivity. Monooctanoin (**17**) is also slower to react, consistent with other investigations including those that show monoglyceride accumulating during lipolysis of triglycerides. All analogues in which *one* of the OH groups of monooctanoin is etherified had the same lower level of activity, and the diether esters **7** and **9** were further reduced in activity. Since this particular lipase is known to be a positionally nonselective enzyme, it was gratifying that the monoether diesters **4** and **13** were equally reactive. However, there appears to be a preference for primary carbinol ester if one compares the relative rate of the di-

**Table III. Reactivities of Selected Esters in the Presence of *C. rugosa* Lipase**

| alcohol | fatty acid carbon no. 4 | 6 | 8 | 9 | 12 | 18:1 |
|---|---|---|---|---|---|---|
| A. Calculated Pseudo-First-Order Rates, $\times 10^3$ (25 °C) | | | | | | |
| glycerol[a] | 1.36 | 0.39 | 8.81 | 3.9 | *b* | 2.50 |
| methanol | 0.12 | 0.07 | 0.58 | 0.90 | 0.14 | 0.59 |
| acetonide of glycerol | 1.90 | 0.38 | 23.0 | 5.05 | 2.89 | 2.59 |
| B. Reactivity Relative to Corresponding Triglyceride | | | | | | |
| glycerol[a] | 1.0 | 1.0 | 1.0 | 1.0 | *b* | 1.0 |
| methanol | 0.09 | 0.18 | 0.07 | 0.23 | | 0.24 |
| acetonide of glycerol | 1.4 | 1.0 | 2.6 | 1.3 | | 1.0 |
| C. Reactivity Relative to Octanoate Ester | | | | | | |
| glycerol[a] | 0.15 | 0.04 | 1.0 | 0.44 | *b* | 0.28 |
| methanol | 0.21 | 0.12 | 1.0 | 1.55 | 0.24 | 1.0 |
| acetonide of glycerol | 0.08 | 0.02 | 1.0 | 0.22 | 0.13 | 0.11 |

[a] The triglyceride was employed. [b] Solid at reaction temperature.

ether esters **7** and **9** (see below). The octanoate ester of the acetonide of glycerol, **14c**, was more reactive than trioctanoin by a factor of 2.6. Several esters of primary alcohols containing rings were also hydrolyzed. A prior study of benzylic, and other, esters catalyzed by porcine pancreatic lipase demonstrated that several oleic acid esters reacted at rates comparable to that of triolein. However, none of the octanoic acid esters examined gave significant reaction rates using *C. rugosa* lipase including the octanoate of cyclopentylmethanol (**21**) which is essentially isosteric with the acetonide ester save for the *gem*-dimethyl group. Although a more exhaustive search for alternate substrates to triglycerides could provide additional useful structures, we chose at this point to examine the relative reactivities of esters of the acetonide of glycerol, **14**.

Homogeneous triglycerides of several fatty acids ($C_4$, $C_6$, $C_8$, $C_9$, $C_{12}$, 18:1) were hydrolyzed with *C. rugosa* lipase with the protocol described above (Experimental Section) and a pseudo-first-order rate constant estimated from the data obtained. The corresponding methyl esters and (2,2-dimethyl-13-dioxolan-4-yl)methanol esters (esters of the acetonide of glycerol), including the hexadecanoate, were similarly hydrolyzed. The data are shown in Table III; Part A presents the calculated initial rates, B gives rates relative to the corresponding triglycerides as 1.0, and C gives rates relative to the octanoates as 1.0. In all instances the acetonides reacted faster than the triglycerides, while the methyl esters reacted more slowly. The data of Table IIIC suggest that if one were to qualify fatty acid selectivity of the commercial *C. rugosa* preparation by the relative reactivities of homogeneous triglycerides, the acetonide esters provide a better comparison than do the methyl esters.

A similar set of hydrolyses were performed with Amano Co.'s Lipase K-30, a nonselective lipase prepared from *A. niger* (Table IVA–C). The acetonide esters were again more reactive than the corresponding triglycerides, while the methyl esters were somewhat less reactive; and the acetonide esters may also serve as the better guide to fatty acid selectivity. Last, a comparison of octanoate esters alone was made with several additional commercial lipases, namely porcine pancreatic lipase, *Rhizopus arrhizus*, NOVO-SP-225 (*Mucor miehei*), Gist-Brocades Lipase-S (*M. miehei*), and Amano-MAP (*M. miehei*) (Table V). The three preparations derived from the, presumably, same fungal species had shown different degrees of stereobias in hydrolyses and esterifications of aliphatic secondary alcohols. (Sonnet and Baillargeon, 1987). This observation can have several possible causes rooted in strain variation, culture production of lipase, precipitation

**Table IV. Reactivities of Selected Esters in the Presence of *A. niger* (Nonselective) Lipase**

| alcohol | fatty acid carbon no. 4 | 6 | 8 | 9 | 12 | 18:1 |
|---|---|---|---|---|---|---|
| A. Calculated Pseudo-First-Order Rates, $\times 10^2$ (25 °C) | | | | | | |
| glycerol[a] | 1.69 | 0.075 | 2.8 | 0.45 | *b* | 0.34 |
| methanol | <0.01 | 0.019 | 0.28 | 0.22 | 0.16 | 0.13 |
| acetonide of glycerol | 2.47 | 0.59 | 4.20 | 0.91 | 3.90 | 0.52 |
| B. Reactivity Relative to Corresponding Triglyceride | | | | | | |
| glycerol[a] | 1.0 | 1.0 | 1.0 | 1.0 | *b* | 1.0 |
| methanol | *c* | 0.25 | 0.10 | 0.49 | | 0.37 |
| acetonide of glycerol | 1.5 | 7.9 | 1.5 | 2.0 | | 1.5 |
| C. Reactivity Relative to Octanoate Ester | | | | | | |
| glycerol[a] | 0.60 | 0.03 | 1.0 | 0.16 | *b* | 0.12 |
| methanol | *c* | 0.07 | 1.0 | 0.79 | 0.56 | 0.45 |
| acetonide of glycerol | 0.59 | 0.14 | 1.0 | 0.22 | 0.93 | 0.12 |

[a] The triglyceride was employed. [b] Solid at reaction temperature. [c] Too slow to provide useful data.

**Table V. Relative Reactivities of Selected Esters in the Presence of Various Commercial Lipases[a]**

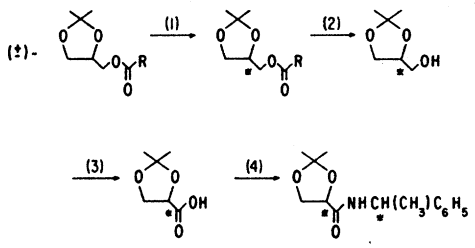
| ester | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| trioctanoin | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| methyl octanoate | 0.10 | 0.07 | 0.05 | 0.21 | 0.50 | 0.06 | 0.04 |
| acetonide octanoate | 1.50 | 2.60 | 0.07 | 0.18 | 1.23 | 0.39 | 0.15 |
| triolein | 1.00 | 1.00 | 1.00 | | 1.00 | | |
| methyl oleate | 0.37 | 0.60 | 0.12 | | 1.28 | | |
| acetonide oleate | 1.53 | 2.81 | 0.12 | | 2.15 | | |

[a] Key: A, *A. niger* (nonselective); B, *C. rugosa*; C, *R. arrhizus*; D, porcine pancreas; E, *M. miehei* (NOVO); F, *M. miehei* (Amano); G, *M. miehei* (Gist-Brocades).

techniques, etc. It was interesting to note that the NOVO lipase that had shown the greatest stereobias was significantly more effective in hydrolyzing methyl octanoate and (2,2-dimethyl-1,3-dioxolan-4-yl)methyl octanoate. Table V provides relative rate data for oleate esters. It appears that the acetonide esters are likely to be considerably more reactive than the corresponding methyl esters and often are even more reactive than the corresponding triglycerides. Since acetonide esters are easily prepared, monofunctional compounds, and the longer chain decanoate and dodecanoate, are liquids, such esters may be suitable substitutes for triglycerides themselves in studies of lipase activity.

The diether esters **7** and **9** were examined to learn whether they might be useful in determining lipase positional selectivity. The usual method for determination involves hydrolyzing specific mixed triglycerides in a manner that must allow cancellation of fatty acid selectivity. This pair of compounds, or analogues, could afford a simple evaluation of selectivity since the corresponding diether glycerols that result from hydrolysis are easily analyzed by HPLC and do not undergo internal rearrangement. A control experiment in which **2a** was heated in 6 N HCl for 16 h yielded only starting material. Lipolyses of **7** and **9** were conducted with *C. rugosa* lipase; percent conversion was determined by free fatty acid titration, and product ratio was obtained by HPLC. These data allowed calculation of an enzyme reaction rate ratio that could be compared to the manufacturer's assignment of selectivity, presumably based on conventional methodology. Judged by the relative rate of hydrolysis of the diether esters, *C. rugosa* demonstrated some positional selectivity. The measured initial reaction velocities of the diether esters **7** and **9** were in a ratio of ca. 2:1 consistent with the competitive experiments. However, as mentioned earlier, the reactivities of the diesters **4** and **13** toward *C.*

**Scheme III. Derivatization Sequence for Configuration Analysis of (2,2-Dimethyl-1,3-dioxolan-4-yl)methanol**[a]



[a] Key: (1) lipase, partial hydrolysis; (2) $NaOCH_3$, $CH_3OH$; (3) $H_2CrO_4$/acetone; (4) $SOCl_2$, DMF; (*S*)-$H_2NCH(CH_3)C_6H_5$.

**Table VI. Enantiomeric Ratios Obtained in Hydrolysis of Acetonide Esters of Glycerol**

| enzyme[a] | ester | C[b] | ee[c] | E[d] |
|---|---|---|---|---|
| *A. niger*[e] | butyrate | 0.500 | 0.55 | 5.8 |
| | octanoate | 0.500 | 0.36 | 3.0 |
| *C. rugosa* | butyrate | 0.500 | 0.14 | 1.5 |
| | octanoate | 0.500 | 0.23 | 2.0 |
| | dodecanoate | 0.408 | 0.04 | 1.2 |
| *M. miehei* | butyrate | 0.500 | 0.16 | 1.6 |
| | octanoate | 0.500 | 0.088 | 1.3 |
| porcine pancreas | butyrate | 0.500 | 0.38 | 3.2 |
| | octanoate | 0.500 | 0.33 | 2.7 |

[a] Reactions were conducted at pH 7.00 and 25 °C (see the Experimental Section). [b] Mole fraction conversion to free fatty acid. [c] Enantiomeric excess (mole fraction enantiomer A − mole fraction enantiomer B). [d] Enantiomeric ratio (see the Experimental Section). [e] Reactions of the butyrate were conducted at various pH (E): 5.2 (ca. 1.0), 6.0 (2.13), 7.0 (4.38), 8.0 (2.36), 9.0 (2.26).

*rugosa* were approximately equal. Additionally, **6** was esterified faster than **8** in companion experiments also using the *C. rugosa* lipase (rate ratio 4.9). Because these compounds that contain one or two *n*-alkyl ether units are considerably slower to react than the triglycerides, the poor comparisons of positional selectivities calculated from the reactions that employed them as substrates are taken as further indication that the *n*-alkyl ether substituent may not be a good substitute for a *n*-alkanoyl, at least not in studies of lipase selectivity.

A particular advantage in employing acetonides of glycerol as pseudolipids is that the asymmetric center can be easily exploited to judge the stereochemistry of the lipase-catalyzed hydrolysis (or esterification, transesterification). Estimations of lipase stereobias in reactions of triglycerides have depended upon rate measurements made on stereochemically pure substrates. Recently, by way of demonstrating the utility of porcine pancreatic lipase in resolving racemic esters, a chiral shift reagent was employed to determine the configurational purity of the acetonide of glycerol (Ladner and Whitesides, 1984). This procedure was applied subsequently in a study involving transesterification with this same alcohol in tributyrin (Sonnet et al., 1986). An alternate analysis that does not require NMR spectrometry was devised in which the alcohol was oxidized to the carboxylic acid (Scheme III); the acid was then transformed via the acid halide to an amide with (*S*)-α-methylbenzylamine. The resulting diastereomeric amides are then analyzed by GLC (see the Experimental Section).

In order to assess stereobias, if any, butanoate and octanoate esters of the acetonide of glycerol were hydrolyzed with several lipases under pH-stat conditons to 50% conversion. The enantiomeric excess of residual ester was determined after transesterification to the alcohol ($NaOCH_3$, $CH_3OH$) and conversion of that alcohol to the diastereomeric carboxamides for analysis. These data

**Table VII. Stereoselectivity of *R. delemar* Lipase**

| source[a] | acetonide ester | C[b] | ee[c] | E[d] |
|---|---|---|---|---|
| Tanabe | butyrate | 0.333 | 0.20 | 2.8 |
| | butyrate | 0.467 | 0.32 | 2.9 |
| | octanoate | 0.478 | 0.24 | 2.1 |
| Miles | butyrate | 0.366 | 0.26 | 3.4 |
| | butyrate | 0.441 | 0.37 | 3.9 |
| | octanoate | 0.500 | 0.28 | 2.3 |
| further purified | butyrate | 0.347 | 0.31 | 5.1 |
| | butyrate | 0.480 | 0.44 | 4.3 |
| | octanoate | 0.499 | 0.27 | 2.2 |

[a] Source (specific activity, μmol of free fatty acid generated $min^{-1}$ per mg): Tanabe food grade (3.85); Miles fine grade (140); pure+ (980). [b] Mole fraction conversion determined by titration for free fatty acid. [c] Enantiomeric excess determined by gas chromatographic analysis of diastereomers (see the Experimental Section and Scheme III). [d] Enantiomeric ratio, or relative reactivities of enantiomers calculated as described in the Experimental Section. *S* enantiomer hydrolyzed faster.

allowed calculation of the lipase enantiomeric ratio *E* (Table VI). Those lipases examined exhibited significant, though low, biases in reactions of these esters. The *sn*-3-glycerol position is the faster reacting site in all cases. The data here are consistent with the previous reports of stereoselection involving the acetonide of glycerol and its butyrate ester (Ladner and Whitesides, 1984). The literature is sparse on the subject of triglyceride lipase stereoselectivity: Porcine pancreatic lipase is reportedly nonselective in this respect (Brockerhof and Jensen, 1974) although a mammalian lipase (human gastric) has shown a selectivity for the *sn*-3-esters of synthetic triglycerides that is of magnitude similar to that observed in the cited studies (Borgström and Brockman, 1984). Interestingly, a lipoprotein lipase from *Pseudomonas aeruginosa* (Amano 3-LPL) reportedly performs useful resolution, i.e., has high selectivity, in hydrolysis of the octanoate ester of (1,3,2-oxazolidon-5-yl)methanol. This is an aza analogue of the acetonide of glycerol, and the faster reacting enantiomer has the same relative configuration as an *sn*-3 triglyceride. Thus, the better substrate configuration for lipases appears to be that which most closely approximates the *sn*-3-glycerol structure. Moreover, lipases apparently do show stereoselection in their reactions with triglycerides. This selectivity may show variation with pH as shown in Table VI. Since the lipase was not a homogeneous protein, it is possible that either the relative contributions by different lipase forms to hydrolysis vary with pH or that several differentially protonated species of the same enzyme that react with slightly different enantiomeric ratios are operative.

The use of chiral substrates to investigate the stereochemical consequences of enzyme-catalyzed reactions can serve as a useful probe for enzyme active site structure. It has been employed ingeniously, for example, in studies of α-chymotrypsin (Mallick et al., 1984) and horse liver alcohol dehydrogenase (Jones and Beck, 1976). Currently, the investigator is faced with an array of enzymatically heterogeneous lipase preparations, each potentially unique in gross properties such as the various selectivities. Preparations from the same fungal species provided by various producers can perform quite differently (Sonnet and Baillargeon, 1987). Determination of lipase stereoselection for acetonide esters might offer a means to more clearly define the enzymatic character of a given preparation, and such analysis could be useful in following the course of enzyme purification and characterizing the purified protein. In related work we have purified one of the lipolytically active proteins of *R. delemar*, a 1,3-positionally selective lipase that is available from several sources and

in varying degrees of purity (Antonian, 1986). In Table VII are given the results of analyses for stereoselection in lipolysis of the butyrate and octanoate esters of the acetonide of glycerol. The Miles fine grade is greater in protein content than the food grade of Tanabe Co. and has greater specific activity. In this instance, the enantiomeric ratio, $E$, is slightly larger also. It is greater yet for enzyme that has been obtained pure as judged by gel and chromatographic techniques and employing the butyrate ester. The value for $E$ of the octanoates has not changed. Perhaps the other lipolytically active species present initially were of similar stereobias, or they were present in small amount. Since the purification procedures afforded distinction between esterase and lipase activity, the data reveal that it is indeed the lipolytically active protein that is responsible for the stereoselection. Prior indications of lipase stereobias in esterification and hydrolysis have been performed with materials that had not been purified to this extent, and usually the stereoselectivities have been observed with compounds that are structurally remote from triglycerides. It now appears that lipases as a class may have a significant degree of stereoselection in reactions involving their natural substrates.

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